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Corresponding Author: Dr. Hirofumi Arisaka, M.D., Ph.D.

Corresponding Author's Institution: Kanagawa Dental College

First Author: Takeo Sugita, D.D.S.

Order of Authors: Takeo Sugita, D.D.S.; Shigeki Sakuraba, M.D., Ph.D.; Yuki Kaku, Ph.D.; Kazuichi Yoshida, D.D.S., Ph.D.; Hirofumi Arisaka, M.D., Ph.D.; Shun-ichi Kuwana, Ph.D.

Suggested Reviewers: John Young
jyoung@howard.edu

Paul Pilowsky
paul.pilowsky@md.edu.au

Christian Gestreau
christian.gestreau@univ-cezanne.fr

Simon Fung
sjfung@ucla.edu

Opposed Reviewers:

09 Oct 2013

Dr. Peter Scheid

Editor, Respiratory Physiology & Neurobiology

Institut für Physiologie, Ruhr-University Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany

Dear Dr. Scheid;

On behalf of all the authors, I would like to ask you to consider our manuscript entitled ”Orexin induces excitation of respiratory neuronal network in the isolated brainstem-spinal cord of neonatal rat” for publication in Respiratory Physiology & Neurobiology as an original research article. All study participants provided informed consent, and the study design was approved by an ethics review board. This manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission. This research was supported, in part, by a Grantin-Aid for Young Scientists (B) from Japan Society for the Promotion of Science. The authors report no conflicts of interest.

Sincerely,

Dr. Hirofumi Arisaka, M.D., Ph.D.
Division of Anesthesiology, Department of Clinical Care Medicine, Kanagawa Dental College, 82 Inaoka-cho, Yokosuka, Kanagawa 238-8580, Japan
Tel: +81-46-822-8803 (Ext. 2375）
Fax: +81-046-822-8842
E-mail: arisakahiro@gmail.com

Abstract

Endogenous neuropeptides orexins (hypocretins) play an important role in the regulation of feeding, drinking, endocrine function and sleep-wakefulness. The orexin neuron projection sites include the rostral ventrolateral medulla of brainstem related to the control of breathing. Previous studies suggest that orexins modulate the central CO_2 response of ventilation during wakefulness in the rodent. These led us to examine effects of orexinergic system on central respiratory control by addition of orexin into the superfusion medium in the isolated brain stem-spinal cord of the neonatal rat in the present study. The application of Orexin B resulted in dose-dependent increases in C4 burst rate via brainstem, not via spinal cord. The increases in C4 burst rate induced concomitant increases in the depolarizing cycle rate of preinspiratory (Pre-I) neurons and inspiratory (Insp) neurons. The rhythmic bursts of C4 and Pre-I neurons finally became to be tonic, although the rhythmic bursts of Insp neurons were maintained. Expiratory (Exp) neurons were also depolarized by the application of Orexin B. Our findings indicate that Orexin B activates central respiratory activity mainly through depolarizing and decreasess in membrane resistance of Pre-I neurons and Insp neurons, and possibly through early start of expiratry phase induced by depolarization of Exp neurons.

Key words: orexin, respiration, central respiratory control, neonate

Highlights

We analyse effects of orexin on central respiratory control using the isolated brainstem-spinal cord preparation of newborn rats. Orexin produces dose-dependent increases in C4 burst rate via brainstem. The increases in C4 respiratory activities are induced by orexin-induced depolarization of pre-inspiratory and inspiratory neurons.

Introduction

Endogenous neuropeptides orexins (hypocretins) play an important role in the regulation of feeding, drinking, endocrine function and sleep/wakefulness (Gastreau 2008). Additionally, both anatomical and functional evidences indicate that orexins are related to the regulation of breathing (Corcoran 2010).

Orexin is synthesized by neurons of the lateral hypothalamus (Sakurai 1998). These Orexin neurons projects to the ventrolateral medulla and phrenic nuclei of the respiratory network with dense fiber (Young JK 2005), indicating that orexin is possibly included in respiratory regulation. In fact, the intracerebroventricular injection of orexin induces increases in ventilation (Zhang et al. 2005). Furthermore, previous studies indicates that orexin-containing axonal projections innervate the spinal cord (Date 2000; Hervieu 2001). However, little is known as to how they work in central respiratory control in brainstem (Gastreau 2008) and how they modulate respiratory activities through the spinal cord.

In the present study, we examined effects of orexinergic system on central respiratory control at the level of respiratory neurons and spinal cord by addition of orexin B into the superfusion medium in the isolated brain stem-spinal cord of the neonatal rat.

Method

All procedures were conducted in accordance with the guidelines of the institutional scientific committee. Data were obtained from 45 neonatal rats (0–4 days old, Wistar rats).

Isolated brainstem–spinal cord preparation

The isolated brainstem–spinal cord preparation has been described in detail elsewhere (Sakuraba et al., 2003). In brief, the rats were deeply anesthetized with diethyl ether, and the brainstem and cervical spinal cord segment C6 were isolated in a chamber filled with oxygenated artificial cerebrospinal fluid (ACSF). The cerebellum and pons were ablated. The preparation was transferred to a 2 ml recording chamber, and fixed, ventral side up, to a silicon rubber base with miniature pins. The preparation was superfused at 25-26 °C with control ACSF equilibrated with a control gas mixture (2% CO_2 in O_2; pH = 7.8) at a flow rate of 4ml/min. This relatively alkaline superfusate allowed the tissue pH of the superficial (<400 μm) medullary layer to be maintained in the physiological range (Okada et al., 1993). The composition of the ACSF was (in mM) 126 NaCl, 5 KCl, 1.25 NaH2PO4, 1.5 CaCl2, 1.3 MgSO4, 26 NaHCO3 and 30 glucose. C4 ventral root activity was recorded using a glass suction electrode, amplified with a conventional AC amplifier (AVH 11, Nihon Kohden, Tokyo, Japan), and integrated (time constant: 100 ms). The signals were recorded on a thermal array recorder and stored on digital tape for subsequent analysis.

The C4 respiratory rate and peak amplitude (Integ. C4) were measured.

Neuronal recording

The intracellular activity of inspiratory (Insp), preinspiratory (Pre-I) and expiratory (Exp) neurons in the superficial (<400μm) rostral ventrolateral medulla (RVLM) was recorded using a perforated patch clamp technique (Kuwana et al., 1998). Neurons were identified and classified on the basis of their firing patterns and the temporal correlation of their activity to the respiratory cycle of C4 ventral root activity (Onimaru et al., 1997). Insp neurons discharge action potentials during the inspiratory phase (C4 burst activity phase) (Fig. 3A). Pre-I neurons are characterized by preinspiratory and postinspiratory action potential discharges and hyperpolarization during the inspiratory phase (Fig. 3B). Exp neurons discharge action potentials between inspiratory phases, and hyperpolarize during the inspiratory phase. A glass pipette (GC100-TF-10, Clark, Reading, UK) was pulled with a horizontal puller (PA-91, Narishige, Tokyo, Japan) to a tip size of approximately 2μm. Electrode resistance ranged from 12 to 16MΩ when the pipette was filled with a solution containing (in mM) 140 K-gluconate, 3 KCl, 10 EGTA, 10 HEPES, 1 CaCl2, 1 MgCl2, and nystatin (100 μg/ml). pH was maintained at 7.2–7.3 using KOH. The micropipette was inserted into the RVLM using a manual hydraulic micromanipulator. Membrane potentials were recorded using a single-electrode voltage clamp amplifier (CEZ 3100, Nihon Kohden, Tokyo, Japan). Neurons were located by applying positive pressure (10–20 cm H2O) inside the pipette. After a gigaohm seal was obtained, the recorded membrane potential gradually became negative, and stabilized in about 10 min. The membrane potential was presented without correcting the liquid junction potential. The resulting perforated patch recording remained stable for more than 60 min.

Protocol

Experiment-1

We analysed the effects of orexin B on C4 respiratory rate and Integ. C4. After the preparation was superfused with control ACSF (CO2 fraction 2%) for at least 30 min and C4 activity reached a steady state, the control superfusate was replaced by a test solution: a control group and three orexin B (Peptide Institute, Osaka, Japan） groups at concentrations 0.01, 0.1, or 1.0μM; each group contained 5 preparations. In the orexin B groups, after obtaining a baseline recording, the superfusate was replaced by solution containing orexin B at the specified concentration, and recording was resumed for 10 min, followed by a washout period using the control ACSF for 10 min. .

Experiment-2

We used a separate perfusion system (Sakuraba et al., 2003) to distinguish the action of orexin B on medulla and spinal cord. The brainstem–spinal cord was separated between the medulla and C1 roots, and separately perfused with control ACSF and ACSF containing 0.1μM orexin B. Ten preparations were randomly allocated to medulla perfusion group or spinal cord perfusion group. Each group contained 5 preparations.

Experiment-3

We analyzed the effect of orexin B on Insp, Pre-I and Exp neurons in the medulla. After the control recording, control ACSF was changed to ACSF containing 0.1μM orexin B for 10 min, followed by a 10 min washout period using control ACSF. Intraburst firing frequencies and resting membrane potential of Pre-I and Exp neurons are represented by the mean firing frequency during the expiratory phase, and intraburst firing frequencies

and resting membrane potential of Insp neurons are represented by the mean firing frequency during the inspiratory phase.

Statistical analyses

All data before and during the application of orexin B were analyzed using the paired *t*-test. A one-way ANOVA with Tukey-Kramer test was used to compare values between different concentration groups. $P < 0.05$ was considered significant. Data are expressed as mean ± SD.

Results

Experiment-1

Integ. C4 burst rates were significantly increased by orexin B 0.1 μM (from 3.80 ± 0.57 to 4.80 ± 0.58 bursts/min; 127.2% ± 13.7% of control; $P < 0.05$; n = 4) and orexin B 1.0 μM (from 4.37 ± 0.47 to 5.90 ± 0.28 bursts/min; 137.3% ± 20.0% of control; $P < 0.001$; n = 6) , however, orexin B 0.01 μM did not (from 4.33 ± 0.42 to 4.25 ± 0.36 bursts/min; 98.4% ± 8.46% of control; n = 6) (Fig.1A-C). These orexin-induced changes of Integ. C4 burst rates were resulted in dose-dependent manner (Fig.1D).

And, of further interest, we found that C4 tonic discharge was induced by the application of orexin B 0.1 μM and 1.0 μM in all preparations (Fig.1B,C). The C4 amplitude did not significantly change in any concentration of orexin B (Fig.1A-C).

Experiment-2

The application of Orexin B 0.1 μM to the medulla, Integ. C4 burst rate significantly increased (from 2.58 ± 0.99 to 4.98 ± 1.21 bursts/min; Fig.2 A; $P <0.01$), however, orexin B 0.1 μM to the spinal cord did not (from 4.04 ± 0.68 to 4.20 ± 1.20 bursts/min; Fig.2 B).

On the other hand, Orexin B 0.1 μM did not induce any significant changes in the the C4 amplitude in both to medulla group (96.1 ± 9.99 % of control; Fig.2 A) and to spinal cord group (104.0 ± 16.4% of control; Fig.2 B). Interestigly, we found that C4 tonic discharge was induced by the application of orexin B 0.1 μM only to spinal cord, not to medulla.

Experiment-3

We analysed the effects of orexin B 0.1 μM on 16 respiratory neurons in RVLM. We classified these neurons into three groups—Insp, Pre-I and Exp—based on their firing patterns during perfusion with control ACSF. The application of orexin B 0.1 μM significantly increased Integ. C4 burst rate from 5.14 ± 1.78 to 7.54 ± 1.99 ($P < 0.0001$; n = 16).

3.1 Effects of NMBAs on Insp neurons and Pre-I neurons

Orexin B 0.1 μM induced a synchronous increases in C4 respiratory rate and rhythmic burst rate in Insp neurons and Pre-I neurons in the early stage of orexin B effects from 5.74 ± 1.65 to 6.98 ± 1.59 ($P < 0.05$; n = 5) and from 5.12 ± 1.36 to 8.05 ± 1.66 ($P < 0.01$; n = 6). Finally, rhythmic C4 burst became to be tonic even though rhythmic burst of Insp neuron and Pre-I neurons were maintained in all preparations (Fig. 3A, B). Also, Orexin B 0.1 μM induced significant decreases in input membrane resistance and depolarization in Insp neurons and Pre-I neurons during orexin-induced respiratory acceleration (Fig. 3A, B; Table).

3.2. Effects of NMBAs on Exp neurons

In Exp neurons, input membrane resistance was slightly decreased by the application of Orexin B 0.1 μM, but the difference was not significant (Fig. 3C; Table; $P = 0.18$), although orexin B 0.1 μM induced significant depolarization in Exp neurons (Fig. 3C; Table; $P < 0.0001$). The rhythmic inhibitory potential cycle rate of Exp neurons was synchronously increased with C4 respiratory rate by the application of orexin B 0.1 μM

(Fig. 3C).

Disscussion

We have demonstrated that orexin B induce dose-dependent increases in C4 respiratory rate in the isolated brainstem-spinal cord preparations of newborn rats, and these increases in C4 respiratory rate are synchronous with increases in depolarizing cycle rate of Pre-I neurons and Insp neurons. Orexin B activates central respiratory activity mainly through depolarizing and decreasess in membrane resistance of Pre-I neurons and Insp neurons, and possibly through early start of expiratry phase induced by depolarization of Exp neurons. Also, we found that orexin B induces C4 tonic discharge with no effects on the amplitude of C4, indicating that orexin B possibly induces direct effects on spinal cord.

In the *in vivo* model, activation of orexin receptors at different levels of the brainstem and spinal cord elicits breathing activity differently. Although stimulation of the hypothalamus (Kayaba Y et al., 2003) and microinjection of orexin into pons induces increases in respiratory frequency (Dutschmann M et al. 2007), microinjection of orexin into the ventrolateral medulla including central respiratory control increases the tidal volume without any effects on respiratory frequency (Young JK et al. 2005; Liu ZB et al. 2010). Contraly, in the isolated brainstem-spinal cord preparations of newborn rats, hypothalamus where orexin is synthesized (Sakurai T et al. 1998) and sends dense fiber projections to many brain regions including central respiratory control (Gestreau C et al. 2008) and pons are ablated (Okada Y et al. 1998). Therefore, it is the most ideal model to analyse the pure effects of orexin on central respiratory control and phrenic activities.

Our result that orexin induces increases in respiratory frequency without any effects on C4 amplitude is contradictory to the above *in vivo* results orexin

microinjected into the ventrolateral medulla increases the tidal volume without any effects on respiratory frequency (Young JK et al. 2005; Liu ZB et al. 2010). In general, removal of the vagal afferent inputs (Onimaru H, 1995) induce changes in respiratory frequency, not amplitude, by the application of some drugs in the isolated brainstem-spinal cord preparations of newborn rats. On the other hand, respiratory stimulator increases only the amplitude of inspiratory activity without vagal feedback in the *in vivo* (Eugenin J et al. 2001). Therefore, our results in the *in vitro* preparation are consistent with the past results in the *in vivo* study.

It is found that projections of orexin-containing neurons to respiratory-related brain stem regions and neurons of the pre-Bötzinger complex express orexin receptors (Young JK wt al. 2005). As mentioned above, it is also found that orexin produces increases in respiratory activities, although respiratory neuronal mechanisms are not analysed. In the present study, we could demonstrate that orexin activates central respiratory activity mainly through depolarizing and decreasess in membrane resistance of Pre-I neurons and Insp neurons, and possibly through early start of expiratry phase induced by depolarization of Exp neurons. Orexin possibly lowers the membrane resistance of these respiratory neurons by opening some channel of respiratory neurons and then induces depolarization of these neurons. However, further studies are needed to analyse these channel mechanisms.

Orexin does not induce any significant changes in the C4 amplitude but C4 tonic discharge is induced by the application of orexin only to spinal cord, indicating that orexin might modulate respiration-unrelated lumbar motoneurons by several pathways. Orexin receptors produce neuroexcitation by post-synaptic depolarization via

activation of non-selective cation channels, inhibition of K^+ chanels, activation of Na+/Ca^{2+}-excahnge and Ca^{2+} influx via L-type Ca^{2+} chanel activation (Kukkonen JP and Leonard CS. 2013; Wu WN et al. 2013). Also, inhibition of hyperpolarization-activated/cyclic nucleotide-gated chasnnels and enhancement of excitation of pyramidal neurons in prelimbic cortex (Li B et al. 2010) might be related to orexin-induced C4 tonic discharges. Further studies are needed to analyse the mechanisms of these orexin-induced C4 tonic discharge.

In conclusion, orexin produces dose-dependent increases in C4 burst rate via brainstem, not via spinal cord. The increases in C4 burst rate induced concomitant increases in the depolarizing cycle rate of Pre-I neurons and inspiratory Insp neurons. The rhythmic bursts of C4 and Pre-I neurons finally became to be tonic, although the rhythmic bursts of Insp neurons were maintained. Exp neurons were also depolarized by the application of Orexin. Orexin activates central respiratory activity mainly through depolarizing and decreasess in membrane resistance of Pre-I neurons and Insp neurons, and possibly through early start of expiratry phase induced by depolarization of Exp neurons.

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Figure legend

Fig.1 Effects of orexin B on the C4 respiratory activity

Representative sample tracing of integrated C4 activity before, during and after superfusion with control and orexin B solution at concentrations 0.01μM (A), 0.1μM (B), and 1.0μM (C). Higher concentrations of orexin B tended to produce significant increase in C4 burst rate in dose-dependent manner (D). *P ＜ 0.05 vs control, **P ＜ 0.05 between different concentration groups)

Fig.2 Effect of orexin B on the medulla and the spinal cord

Representitive sample tracing of integrated C4 activity before, during and after separate superfusion with 0.1μM orexin B solution (A: medulla, B: spinal cord). C4 burst rate increases after application of orexin B 0.1μM (A). C4 tonic discharge were observed in all preparation of spinal cord groups (A), however, it was not observed in any preparation of medulla groups (B).

Fig.3

Representitive sample tracing of inspiratory neuron (A), preinspiratory neuron (B) and expiratory neuron (C) with integrated C4 activity before, during and after superfusion with 0.1μM orexin B solution.

Figure

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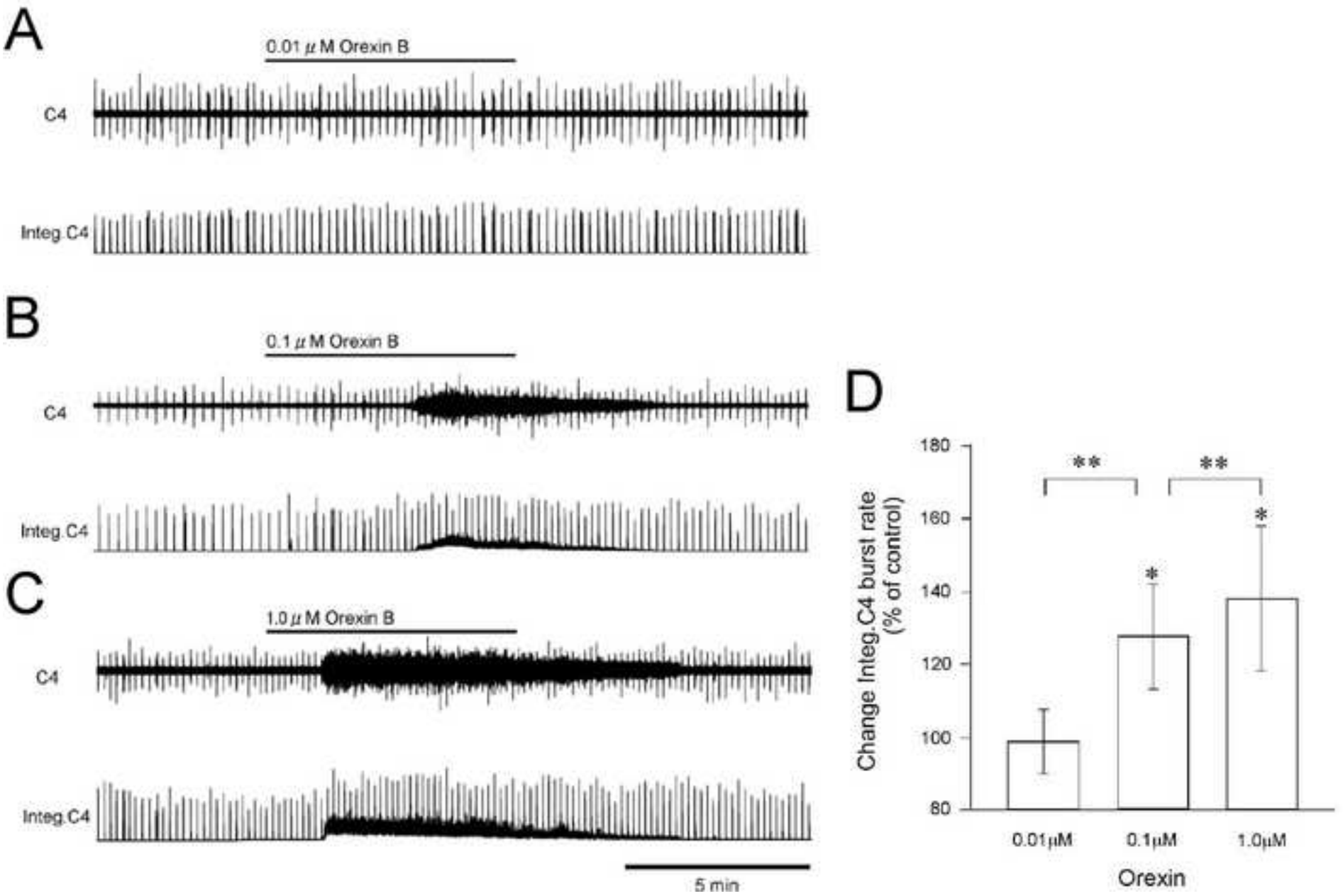

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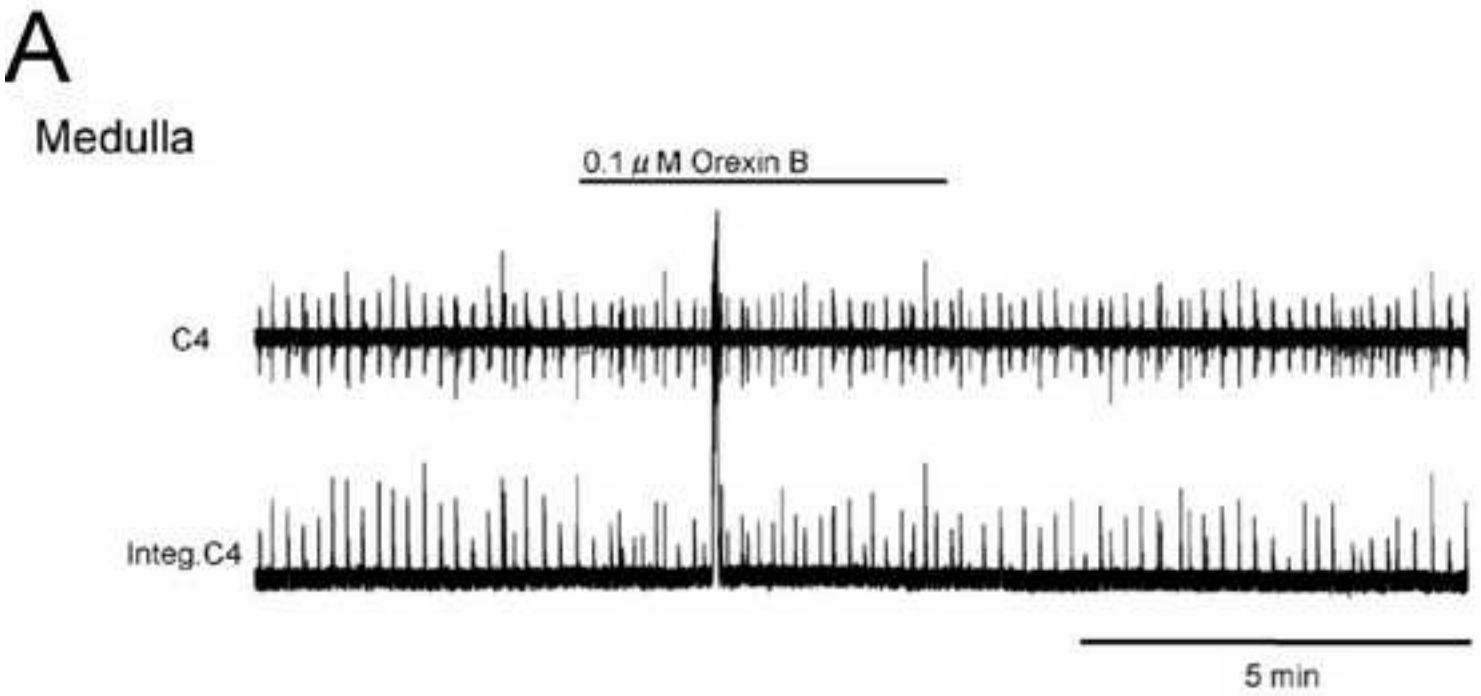

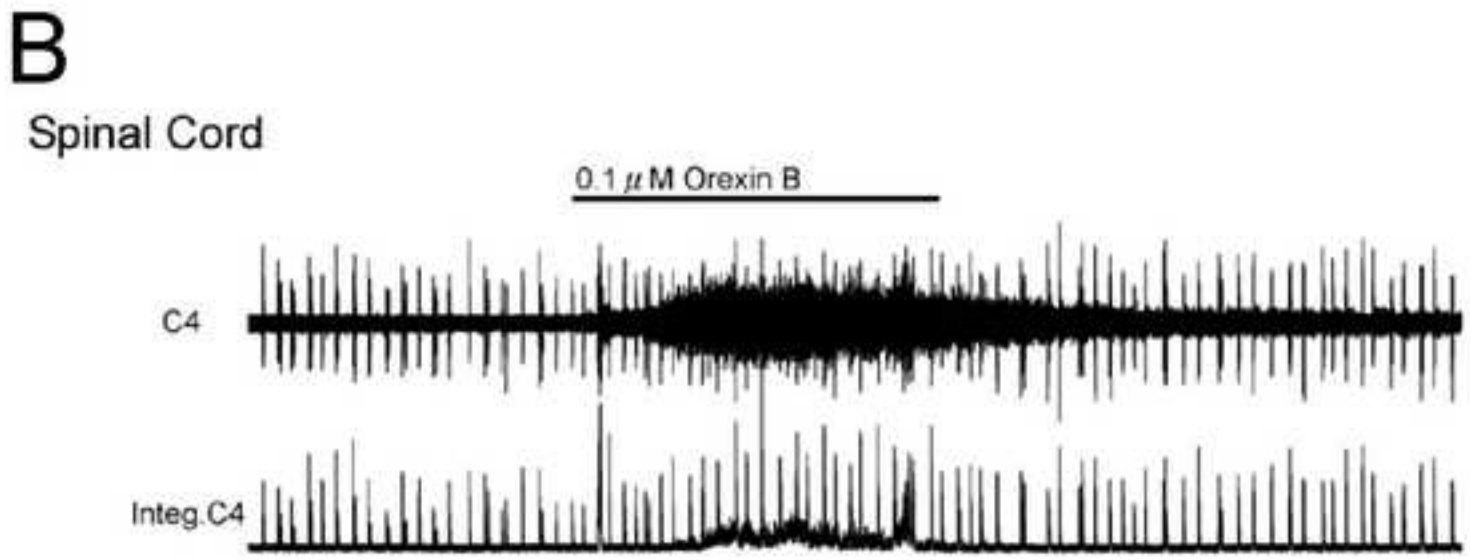

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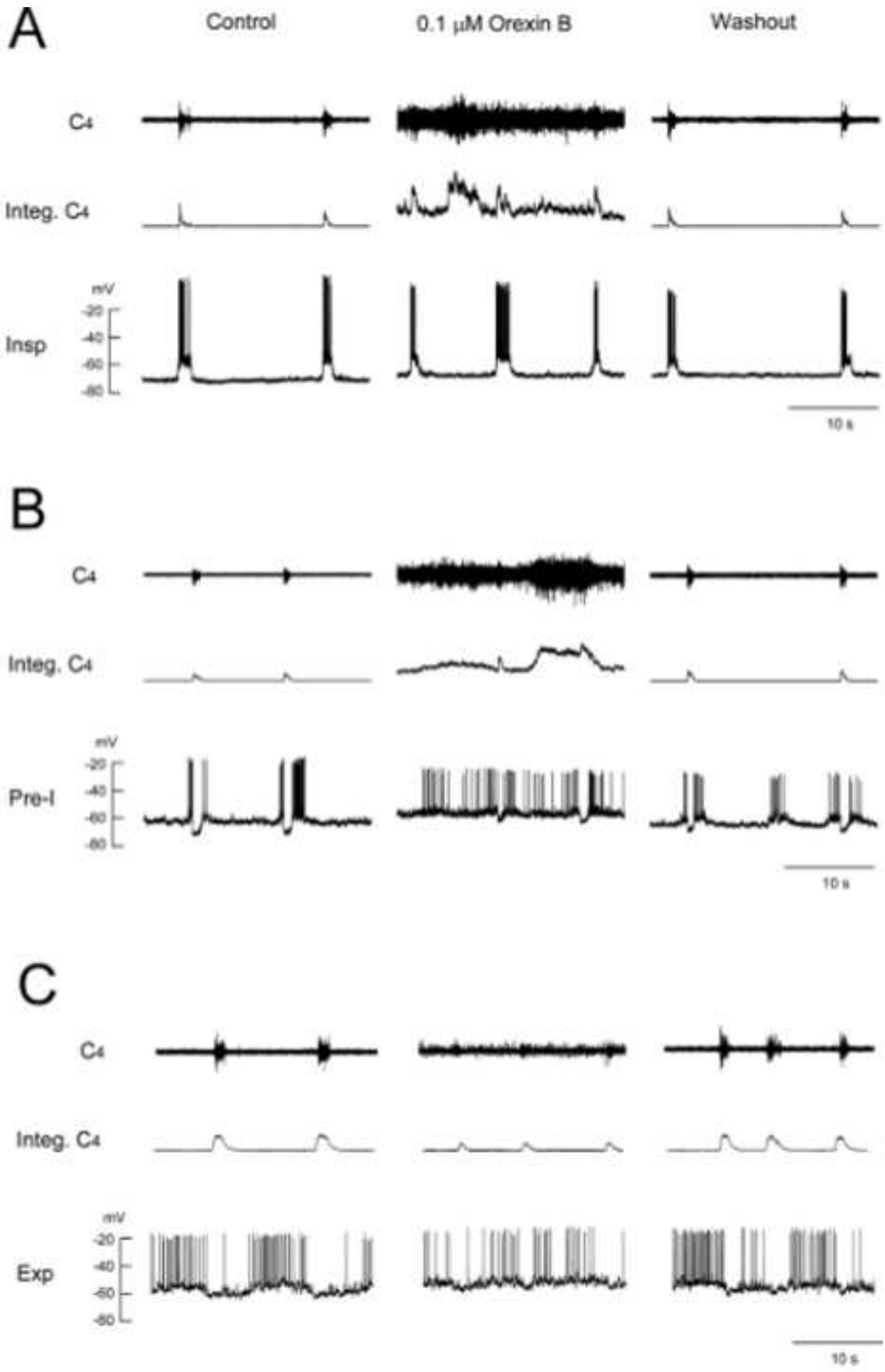

Table

Table

Effects of orexin B on resting membrane potential and membrane resistance of respiratory neurons

		control	0.1μM Orexin
Insp neurons (n = 5)	Em (mV)	-63.6 ± 18.1	-56.4 ± 17.1**
	Ri (MΩ)	484.0 ± 185.1	322.0 ± 113.2*
Pre-I neurons (n = 6)	Em (mV)	-62.0 ± 7.7	-55.3 ± 8.0**
	Ri (MΩ)	540.0 ± 207.8	426.7 ± 206.9**
Exp neurons (n = 5)	Eml (mV)	-55.4 ± 19.1	-48.2 ± 20.0***
	Ri (MΩ)	640 ± 306.9	494.0 ± 251.2

Em: resting membrane potential; Ri: input membrane resistance

* $P < 0.05$

** $P < 0.01$

*** $P < 0.0001$